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Carbon Nanotube Delivery of the GFP Gene into Mammalian Cells

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Exogenous-gene expression and manipulation in mammalian cells has become a mainstay of biomedical research. Consequently, improving methods for efficient gene transfer to a broad range of cell types is of great interest and remains a high priority.[1] Several classes of transfection methods have been developed, which include traditional cationic molecule-mediated agents,[2] such as Lipofectamine 2000™[3] and FuGENE 6™,[4] viral-vector systems,[5] and the "gene gun" approach.[6] With the rapid development of nanobiotechnology, a variety of new materials, such as gold nanoparticles,[7] silica nanoparticles,[8] polymers,[9] nanogels,[10] and dendrimers[11] have been investigated as biocompatible transporters.

Recently, carbon nanotube—a well-studied nanomaterial—have been investigated for their ability to interact with and affect living systems. For instance, carbon nanotubes have been found to enhance DNA amplification in PCR[12] and affect the growth pattern of neurons.[13] Pantarotto et al. have reported the internalization of fluorescein isothiocynate (FITC) labeled nanotubes[14] and nanotube delivery of the gene that encodes β-galactosidase[15] into cells, with no apparent toxic effects. Kam et al. have studied the mechanism of protein-conjugated carbon nanotube uptake into cells via the endocytic pathway.[16,17]

Here we present our finding that amino-functionalized multiwalled carbon nanotubes (NH_2-MWCNTs) are able to interact with plasmid DNA and deliver the green fluorescence protein (GFP) gene into cultured human cells. Our data strongly suggest that carbon nanotubes can be considered as a new carrier for the delivery of biomolecules, such as DNA, proteins, and peptides into mammalian cells. Therefore, this novel system might have potential applications in biology and therapy, including vaccine and gene delivery.

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In order to increase their biocompatibility, we introduced amino-, carboxyl-, hydroxyl-, and alkyl groups onto the surface of MWCNTs. COOH-MWCNTs were first prepared by nitric/sulfuric acid oxidation, and then NH_2- and $CH_3CH_2CH_2$-groups were added. Finally, we obtained four types of MWCNTs with different chemical groups on their surface. Functionalized MWCNTs were observed under an electron microscope and were found to be 60–70 nm in diameter and 1–2 μm in length. Although we did not find a significant difference in size between the NH_2-MWCNTs and NH_2-MWCNT–DNAs, the latter appeared to have the tendency to aggregate (Figure 1 B).

In order to test the DNA-binding ability of amino-, carboxyl-, hydroxyl-, and alkyl-group-modified MWCNTs, we incubated them with pEGFPN1-plasmid DNA, and MWCNT–DNA mixtures were analyzed by agarose-gel electrophoresis. The results show that only NH_2-MWCNT bound to DNA (Figure 2); since the NH_2-MWCNT–DNA complex was too big to run into the

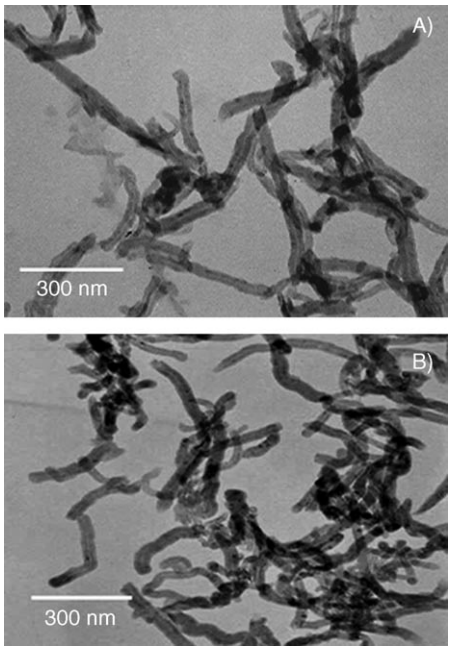

Figure 1. TEM images of A) NH_2-MWCNTs and B) NH_2-MWCNT–DNA.

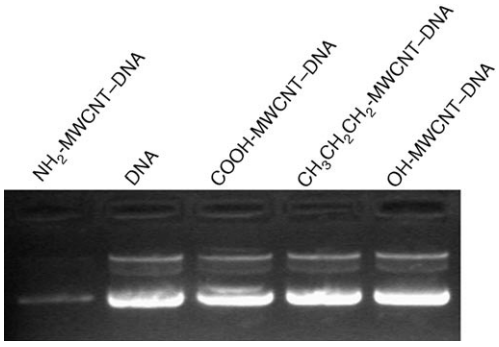

Figure 2. The interaction between plasmid DNA and MWCNTs that were modified with different chemical groups was analyzed by agarose gel electrophoresis.

gel, only a small amount of unbound DNA was visualized with ethidium bromide staining. However, the carboxyl-, hydroxyl-, and alkyl-modified MWCNTs did not form a complex with pEGFPN1 plasmid, and therefore a larger amount of free DNA could move into the gel.

To show whether DNA binding by NH_2-MWCNTs was due to an electrostatic interaction between the positively changed NH_2-MWCNT and negatively charged plasmid DNA, we quantified the concentration of amino groups on the surface of the nanotubes by using the Kaiser assay. The concentration of amino groups on NH_2-MWCNTs was found to be about 0.934 mmol g^{-1}; this suggests that there is a high positive-charge density on the surface of the NH_2-MWCNTs. Therefore we designed different charge ratios of NH_2-MWCNT–DNA for the following experiments.

We performed scanning-electron microscopy (SEM) to visualize the interaction of NH_2-MWCNTs with human umbilical vein endothelial cells (HUVEC). Cell cultures were first incubated with NH_2-MWCNTs and then fixed for SEM analysis. As shown in Figure 3 A, we observed that a lot of NH_2-MWCNTs bound to the cell surface. This binding might be due to the interaction between the positive charge of NH_2-MWCNTs and the negative charge of the cell membrane.

This interaction was further confirmed by transmission-electron microscopy (TEM; Figure 3 B). We found that most NH_2-MWCNTs attached to the cell surface but only few were internalized into the cytoplasm (Figure 3 B, arrows).

In order to determine whether functionalized MWCNTs could be used for gene delivery into HUVEC cells, we used the pEGFPN1 plasmid, which contains a GFP reporter gene. Lipofectamine 2000 was used in this study as a positive control. Therefore, MWCNT–pEGFPN1 mixtures were introduced into HUVEC and A375 (a human melanoma cell line) cell cultures. The cultures were incubated for a further 48 h in order to allow for GFP-gene expression, and the transfected cells were then imaged under a fluorescence microscope. The transfection efficiency was evaluated by calculating the percentage of the cells with green fluorescence. As shown in Figure 4, we found that NH_2-MWCNTs, like Lipofectamine 2000, efficiently delivered the exogenous GFP gene into cells, and the protein was successfully expressed in both HUVEC (Figure 4 A) and A375 cells (data not shown). Fluorescence was not observed with control cells that were incubated either with pEGFPN1 (alone) or carboxyl-, hydroxyl-, or alkyl-modified MWCNTs (Figure 4 B). The transfection level of NH_2-MWCNTs was lower than that of Lipofectamine 2000.

To optimize the proportion of NH_2-MWCNTs to DNA, we made a series of NH_2-MWCNT–pEGFPN1 mixtures at different charge ratios: 10:1, 10:2, 10:3, 10:4, 10:5, and 10:10 (+:−). Cells were then incubated with each NH_2-MWCNT–DNA preparation in DMEM medium for 48 h. Subsequently, we found that the NH_2-MWCNT–DNA mixture that was prepared at a charge ratio of 10:2 (+:−) showed the highest GFP-transfection efficiency, although this was still lower than that of Lipofectamine 2000 (Figure 4 C).

In order to determine whether the lower transfection efficiency of NH_2-MWCNTs was due to its cytotoxicity, we performed MTT assays (Figure 5). NH_2-MWCNTs did not affect cell growth even at a high concentration (60 μg mL^{-1}). However, Lipofectamine 2000 was found to be toxic to cells at concentrations above 20 μg mL^{-1}. These data demonstrate that NH_2-

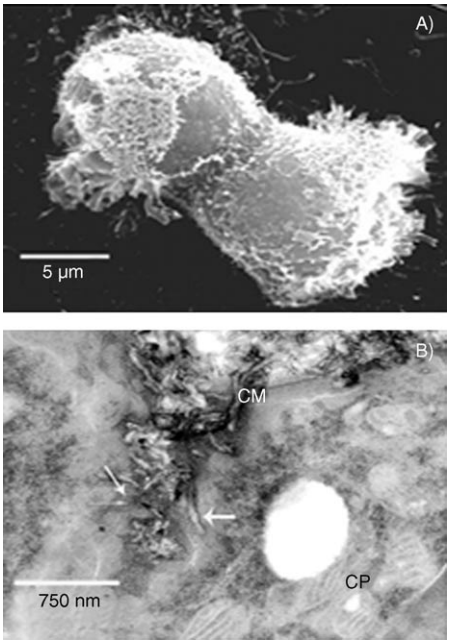

Figure 3. A) SEM and B) TEM images showing the interaction between NH_2-MWCNTs and HUVEC cells. CP: cytoplasm; CM: cell membrane.

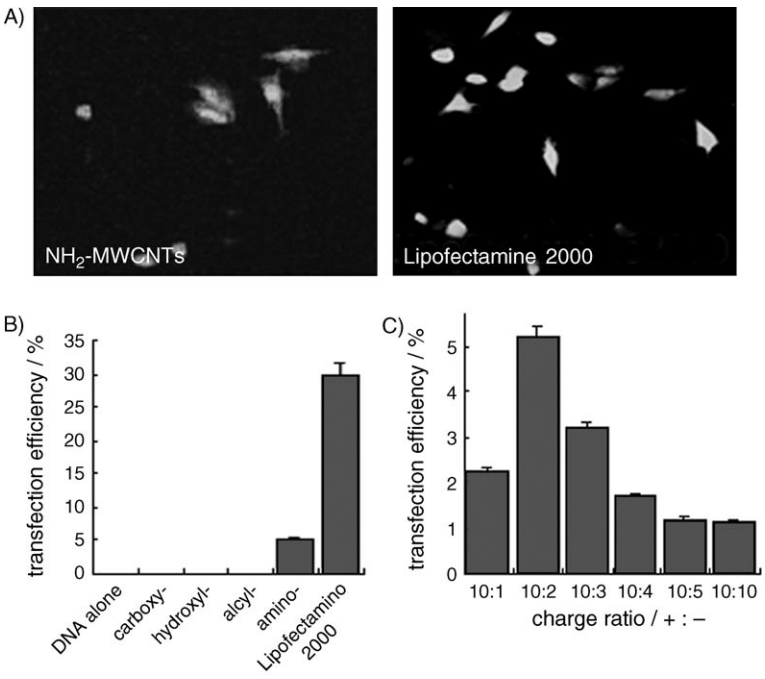

Figure 4. A) Fluorescence microscopy of HUVEC cells that were transfected with GFP either by using NH_2-MWCNTs or Lipofectamine 2000 (200× magnification). B) Evaluation of the transfection efficiency of pEGFPN1 plasmid by using MWCNTs that were modified with different chemical groups. C) Transfection efficiency of NH_2-MWCNT–pEGFPN1 samples that were prepared at different charge ratios (+:−).

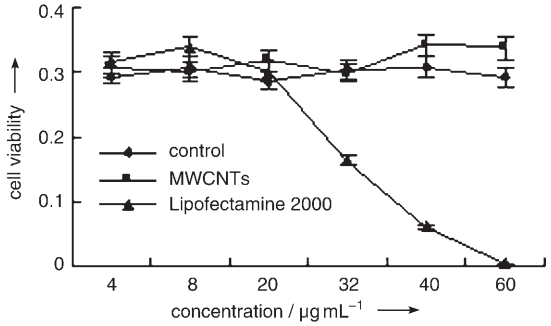

Figure 5. Cytotoxicity of different concentrations of NH_2-MWCNTs and Lipofectamine 2000 in HUVEC cells.

MWCNTs have little or no cytotoxicity compared with Lipofectamine 2000; this is highly advantageous in gene transfection.

In this study, we found that the nature of the chemical groups on the surface of carbon nanotubes is a critical factor that can affect transfection efficiency. We modified the surface of MWCNTs with several chemical groups including amino-, carboxyl-, hydroxyl-, and alkyl groups, and evaluated the tubes' ability to deliver the GFP gene into HUVEC and A375 cell lines. The results show that NH_2-MWCNTs, but not carboxyl-, hydroxyl-, or alkyl-modified MWCNTs, are able to deliver the pEGFPN1 plasmid into cells. This result suggests that the positive charge introduced with an amino group might allow NH_2-MWCNTs to bind to anionic plasmid DNA and cell membrane through electrostatic interactions; this could be the initial step for gene transfection.

We used MWCNTs to deliver the GFP gene, unlike other studies, which have used the genes for β-galactosidase and luciferase.[18] Although the transfection level of NH_2-MWCNTs was lower than that of Lipofectamine 2000, little or no cytotoxicity towards mammalian cells was observed. This is important because many transfection reagents have high toxicity. Compared with single-wall carbon nanotubes (SWCNTs), MWCNTs have many advantages: they can be produced on a larger scale, at lower cost, can be easily functionalized, and are biocompatible. Several studies have demonstrated that MWCNTs can be used with a broad range of cell lines, including HeLa cells, CHO cells,[15] and HUVEC and A375 cells in this study. This suggests that MWCNTs can become a new promising reagent for gene delivery. Although the exact mechanism by which NH_2-MWCNTs carry genes into cells is still unclear, Kam et al. have suggested that carbon-nanotube conjugates might be taken up by the endocytic pathway.[16]

The possibility of improving the transfection efficiency of nanotubes with shorter or smaller size, and higher-charge density has also been discussed previously.[19,20] Further elucidation of the mechanism of cellular uptake of DNA–nanotube complexes will contribute to the optimization of carbon nanotubes—in terms of their length, diameter, charge density, and solubility—as vehicles for gene delivery.

We functionalized MWCNTs with several chemical groups and found that only positively charged NH_2-MWCNTs could bind to anionic plasmid DNA and deliver it into mammalian cells. Furthermore, we have shown the interaction between NH_2-MWCNTs with the cell surface by SEM imaging. Little or no cytotoxicity was observed with NH_2-MWCNT-mediated gene transfer. These findings suggest that the positive charge of the amino group on NH_2-MWCNTs is a critical factor for gene delivery and that these functionalized nanotubes could be used in biological research and gene therapy.

Experimental Section

Modification of MWCNTs with different chemical groups: MWCNTs, which had been prepared by the chemical-vapor deposition (CVD) method, were purchased from Sun Nanotech Co. Ltd. (Nanchang, China). They were purified with concentrated nitric acid before chemical modification.

COOH-MWCNTs were first prepared by sonication in a dilution of concentrated nitric aid/sulfuric acid (1:3, *v/v*) at 50 °C for 24 h. In order to introduce the amino group, COOH-MWCNTs were stirred in freshly distilled thionyl chloride at 70 °C for 24 h. They were then mixed with excess hexane-1,6-diamine and stirred at 60 °C for 48 h. For the introduction of the alkyl group, COOH-MWCNTs were sonicated in NaOH (5 mM) for 2 min and mixed with tetra-*n*-octylammonium bromide (TOAB) and $CH_3(CH_2)_2Br$ and vigorously stirred for 2 h.[21] After the mixture had been thoroughly washed with deionised water and dried under vacuum at 50 °C, COOH-MWCNTs, NH_2-MWCNTs, and $CH_3(CH_2)_2$-MWCNTs were obtained.

To make OH-MWCNTs, purified MWCNTs were treated with potassium hydroxide.[22] Typically, MWCNTs/potassium hydroxide (1:20, *w/w*) was treated in a stainless steel capsule that contained a milling ball. This was then vigorously shaken for 2 h in air at room temperature by using a Wig-L-Bug (Bratt 3110-B). After the mixture had been thoroughly washed with deionised water and dried under vacuum at 50 °C, we obtained OH-MWCNTs.

For the following experiments stock solutions of carboxyl-, amino-, hydroxyl-, and alkyl-MWCNTs were prepared in deionized water (5 mg mL^{-1}). To make sure that the carbon nanotubes were homogeneously dispersed, the stock solutions were ultrasonicated for 5 min before use. The pEGFPN1 plasmid (Clontech, Palo Alto, CA, USA) was dissolved in deionized water (1 mg mL^{-1}).

Kaiser assay: The concentration of amino groups on NH_2-MWCNTs was calculated by using the quantitative Kaiser assay. A standard curve was first constructed with glycine. NH_2-MWCNTs were mixed with acetate buffer (200 μL, 1 M, pH 4.5) and ninhydrin buffer (200 μL; 150 mg ninhydrin, 10 mL isopropanol, 0.3 mL acetic acid, made to the final volume of 100 mL with water). Mixtures were incubated at 100 °C for 20 min and centrifuged at 10 000 rpm for 10 min. The supernatant was analyzed spectrophotometrically at 565 nm. The concentration of amino groups on NH_2-MWCNTs was calculated by using the standard curve obtained with glycine.

Transmission-electron microscopy: For imaging the structure of MWCNTs, NH_2-MWCNTs, and NH_2-MWCNT–DNA, samples were dispersed in deionized water and deposited onto a copper grid that was coated with a carbon support film. After the excess water had evaporated, the copper grid was observed by using a JEM-100CX electron transmission microscope at 80 kV.

In order to image the interaction of NH_2-MWCNTs with cells, HUVEC cells were incubated with NH_2-MWCNTs (final concentration 50 μg mL^{-1}) for 2 h at 37 °C. Then the cells were digested with trypsin (0.05 %, *w/v*) and washed with PBS. They were fixed with gluta-

raldehyde (2.5%, *v/v*) for 2 h and then with a solution of OsO_4 (1%, *w/v*) for 30 min at room temperature. The fixed cells were dehydrated through an ethanol series (50, 70, 90, 100, 100%) and were incubated for 5 min in each solution. Samples were embedded with Epon 812 resin and incubated at 35 °C, 45 °C, and 60 °C for 12 h at each temperature, in sequence. The complex containing the resin and cells was cut into 120 nm thick slices, which were deposited on a carbon grid and observed by using a JEM-100CX electron transmission microscope at 80 kV.

Gel electrophoresis: MWCNTs (2 μL) with the different chemical-group modifications were incubated with pEGFPN1 plasmid (0.6 μL) for 30 min at room temperature. The mixtures were then centrifuged at 10000 rpm for 10 min. The supernatants that contained the free DNA were loaded on an agarose gel (1%, *w/v*) for electrophoresis (10 $V\,cm^{-1}$) and visualized by ethidium bromide (5 $\mu g\,mL^{-1}$) staining.

Scanning-electron microscopy: HUVEC cells were cultured on a cover slip and incubated with NH_2-MWCNTs (final concentration 50 $\mu g\,mL^{-1}$) for 2 h at 37 °C. The cover slip was removed from the growth medium and washed with PBS. After being fixed in glutaraldehyde (2.5%, *v/v*) for 30 min and a further 3 min in OsO_4 (1%, *w/v*), the cells were dehydrated through an ethanol series (50, 70, 90, 100, 100%) for 5 min in each solution, and critically point dried from ethanol. The cells were mounted on specimen stubs with conductive paint and coated with a layer of Au (10 nm thick) in a sputter coater. Cells were viewed through a lens on a Hitachi S-5200 scanning-electron microscope in slow-scan mode at 10 kV accelerating voltage and 0 to ~0.3 mm working distance.

Delivery of GFP gene into human cells: When HUVEC cells had reached 80% confluence, they were incubated with DNA bound amino-, carboxyl-, hydroxyl-, or alkyl-modified MWCNTs (10 μL in DMEM medium) or with pEGFPN1 (3 μL in deionized water) for 30 min, at 37 °C. After 3 h, the culture medium was renewed, and the cells were washed in PBS and then incubated with DMEM containing antibiotics (500 $IU\,mL^{-1}$ penicillin and 500 $\mu g\,mL^{-1}$ streptomycin) and fetal bovine serum (FBS, 10%) and incubated for 48 h at 37 °C with 5% CO_2 in a water-jacketed CO_2 incubator (Forma Scientific Inc. Marietta, OH, USA). A series of NH_2-MWCNT–pEGFPN1 samples with different charge ratios was also prepared (10:1, 10:2, 10:3, 10:4, 10:5, and 10:10; +:−) and tested under the same experimental conditions. GFP-transfected cells that emitted fluorescence were imaged and counted under a fluorescence microscope (excitation at 488 nm, emission at 525 nm). The transfection efficiency was calculated as the percentage of fluorescent cells out of the total number of cells. For the positive control, Lipofectamine 2000 (2 μL, 1 $mg\,mL^{-1}$) and pEGFPN1 (3 μL) at a charge ratio of about 10:3 (+:−) were incubated with the cells in DMEM for 3 h. Then the culture medium was renewed, and the cells were incubated in DMEM with FBS and antibiotics for 48 h, as above. For the negative controls, pEGFPN1 (3 μL) and MWCNTs were incubated directly with the cells.

MTT assay: HUVEC cells were cultured in 96-well plates with DMEM that contained antibiotics (500 $IU\,mL^{-1}$ penicillin and 500 $\mu g\,mL^{-1}$ streptomycin) and FBS (10%). When the cells had reached 80% confluence, NH_2-MWCNTs were added to the following final concentrations: 4, 8, 32, 40, and 60 $\mu g\,mL^{-1}$. After 12 h incubation, MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide; 1 $mg\,mL^{-1}$) was added to the wells and incubated with the cell for 4 h at 37 °C. Then the cells were washed with PBS and incubated with DMSO (80 μL) at room temperature for 3 min and analyzed at 595 nm in a BIO-RAD model 550 microplate reader (Bio-Rad Laboratories, Inc. Hercules, CA, USA). Lipofectamine 2000 was tested under the same conditions. Cells incubated without MWCNTs or Lipofectamine 2000 were used as the negative control.

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